A STUDY OF THE PERMEABILITY OF GRADED NITROCELLULOSE MEMBRANES TO MALT AMYLASE

DISSERTATION

Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in
the Faculty of Pure Science,
Columbia University

BY

CORNELIA TYLER SNELL

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A STUDY OF THE PERMEABILITY OF GRADED NITROCELLULOSE MEMBRANES TO MALT AMYLASE

Introduction

Semipermeable membranes have been used for the study of diffusion phenomena since 1855.[1] Several methods of securing membranes having regular gradations have been employed, in order to control the permeability with respect to certain solutes. In one method the concentration of nitrocellulose in the collodion is varied.[2] The time of evaporation of the solvent may be varied regularly,[3] or evaporation may be allowed to proceed until the membrane reaches a definite weight.[4] The ratio of alcohol to ether in the collodion may be varied.[5] Different amounts of substances other than alcohol and ether, such as glycerol[6] or ethylene glycol[7] may be used in preparing the collodion. Air-dried membranes may be graded by immersion for a definite time in varying concentrations of alcohol before transferring them to water.[8] These methods are all directed toward the same end, that is, a control of the ratio of swelling agent to nitrocellulose in the membrane at the time of immersion in water. Nitrocellulose swells markedly in alcohol, and to a much lesser extent in ethylene glycol. Increasing amounts of swelling agent present at the moment of immersion in water cause increased imbibition of water by the membranes and greater permeability. By the above methods of preparation it has been shown that the permeability of a membrane varies directly with the amount of swelling agent present either in the collodion,[5, 7] or in the first washing liquid.[8] It varies inversely with the concentration of nitrocellulose used,[2] and with the extent of evaporation of the solvent.[3, 4]

The usefulness of graded membranes lies in the fact that they permit fractional dialysis. Certain solutes pass through while others do not. The behavior of a particular substance depends on the grade of membrane used.

Brown[8] obtained membranes covering a wide range of permeability. His least permeable membranes permitted sodium and ammonium chlorides to diffuse through them but not potassium permanganate. A number of substances were used and the grade of membrane reported through which each just failed to pass. The more permeable membranes permitted the passage of starch, ani-

line blue, litmus and Congo Red, but prevented that of Night Blue. Brown demonstrated the possibility of separating two crystalloids or two colloids from each other by means of dialysis.

Eggerth[5] studied the rate of diffusion through graded membranes. The rate of diffusion of a particular substance was found to increase with an increase in the permeability of the membrane. Through the same grade of membrane Congo Red diffused somewhat more rapidly than hemoglobin. Pierce[7] prepared a series of graded membranes and indicated the point in the series at which they became permeable to hemoglobin.

Nelson and Morgan[4] used graded membranes in the purification of invertase by ultrafiltration. They obtained invertase in the filtrate, which was nearly water-white, while highly colored impurities were retained in the residue. Taylor, Braun and Scott[9] employed graded membranes in the electrodialysis of insulin solutions. They obtained a sediment which appeared to be twice as active physiologically as the original. Taylor and Iddles[10] reported the separation of α-amylose from β-amylose, both by ultrafiltration and electrodialysis. α-Amylose was obtained as a hard light brown material, β-amylose as a fine white powder.

Michaelis made extensive diffusion studies with dried nitrocellulose membranes, the permeability of which is very low. Membranes were obtained impermeable to hydrochloric acid when dialyzing against water.[11] In further experiments[12] acetone and urea were found to pass through a particular grade of membrane much more rapidly than glycerol. Glycerol diffused much faster than glucose.

Dialysis is ordinarily introduced at one or more stages in the process of purification of the enzyme, malt amylase. It may be applied to the original extract of ground malt,[13] to a solution of the precipitate obtained by treating the extract with ammonium sulfate[14] or to both,[15] as well as in other ways. Sherman and Schlesinger[28] obtained diffusates which showed diastatic activity, in connection with their work on the purification of malt amylase. If the membranes used in the purification of malt amylase varied considerably in permeability this might lead in some cases to a loss of enzyme material and hence be an important cause for variation in the results obtained in the purification process.

In the work described in this paper graded nitrocellulose membranes were made in order to study quantitatively their permeability to malt amylase as well as some of the factors affecting this. The method of grading chosen was the introduction of varying amounts of ethylene glycol[7] into the collodion. The ethylene glycol was furnished by the Carbide and Carbon Chemicals Cor-

poration. The densities of the two samples used were determined and found to be $1.1098^{25°/4°}$ and $1.1104^{25°/4°}$, as compared with $1.10970^{25°/4°}$, determined by Taylor and Rinkenbach.[16] The refractive index of each of the two samples was 1.4270, determined at 34° C., or 1.4298, calculated to 25° C. by means of the data of Eykmann.[17] The refractive index, as determined in the laboratory of the Carbide and Carbon Chemicals Corporation was 1.430 at 25° C.[18] The nitrocellulose used in preparing collodion was du Pont's "Parlodion." This contains 12.4± 0.1 per cent of nitrogen on a dry basis.[19]

Nitrocellulose Membranes

Preparation of Membranes

The method is essentially that of Pierce,[7] with a somewhat shortened procedure. Throughout this work the concentrations of nitrocellulose and of alcohol in the collodion were kept constant. Varying amounts of ethylene glycol were added to 25 cc. of absolute alcohol and the whole diluted to 100 cc. with anhydrous ether. The 100 cc. of solvent were poured on to 2 grams of nitrocellulose, which had been air-dried, then stored in a dessicator in a refrigerator. The moisture content of the nitrocellulose as used, was about 0.2 per cent. After a gradual swelling process over a period of several hours the nitrocellulose finally goes into solution on shaking.

The per cent by volume of ethylene glycol in the solvent is used to designate the *grade* of the resultant membrane. A membrane made from collodion containing 4 cc. of ethylene glycol in 100 cc. of solvent is called a membrane of grade 4.

In making membranes, 6 cc. of collodion were pipetted on to a polished glass plate 9 cm. in diameter. The glass plates were precisely ground so that both faces were flat and parallel. Four membranes were prepared at a time. The evaporation chamber and other equipment were those used by Field.[20] The chamber was similar in general design to that of Pierce.[7] Air was aspirated through the chamber for a period of 6 hours at the rate of 50± 1 cc. per minute. The air passed through 8 mesh granules of anhydrous calcium chloride at the entrance and exit to the chamber. Fresh calcium chloride was used each time at the exit. The temperature was that of the room, as a rule it varied from 22 to 25° C.

At the end of the 6 hour evaporation period the chamber was opened, one membrane was covered immediately with a watch glass and the other three were transferred on their plates to distilled water at 22-25° C. The covered membrane was weighed

as quickly as possible to get the weight of nitrocellulose plus organic solvent present. This is designated as the *organo-weight*. It was then immersed in water with the others. When the membranes had floated free from the plates they were put in individual dishes of distilled water and let stand over night.

After washing, the wet weight was obtained by weighing a membrane in a covered weighing bottle after blotting off the surface moisture with filter paper. The dry weight, or the weight of the nitrocellulose, was obtained by heating a membrane in an oven at 100° C. for an hour or more, when constant weight was obtained. The dry weights of the membranes averaged about 112 mg. The membranes were stored under water, in which manner they may be kept for several weeks without alteration in properties.

Grading by means of Ethylene Glycol

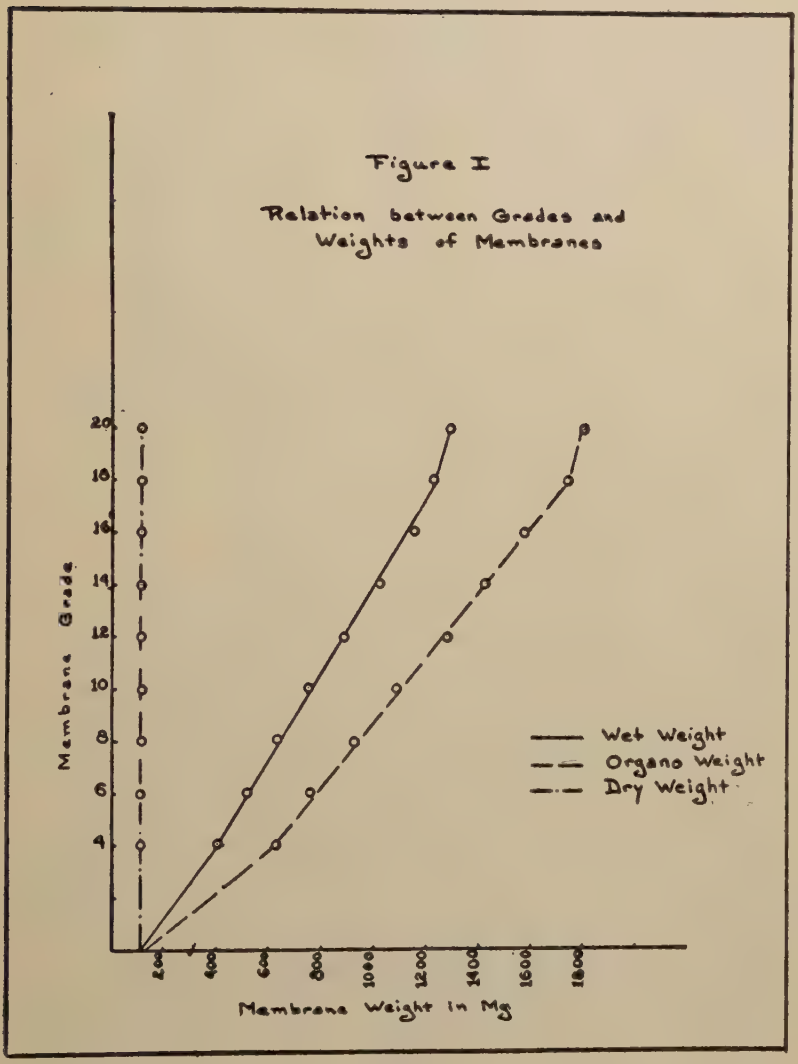
The relatively high wet weights and corresponding permeability of the membranes prepared under the conditions described are due to the presence of ethylene glycol in the collodion. Since the vapor pressure of ethylene glycol is negligible at room temperature,[21] it remains in contact with the nitrocellulose during the evaporation period. It retains with it a part of the alcohol.[20] If no ethylene glycol were present, practically all of the alcohol would evaporate with the ether. Membranes made from collodion containing as solvent 25 cc. of alcohol to 75 cc. of ether and no ethylene glycol had wet weights of about 124 mg., and dry weights of about 114 mg., an increase in the wet over the dry weight of only 9 per cent. The lowest grade of membrane made with ethylene glycol was grade 4.* Grade 4 membranes had an average wet weight of 402 mg., with dry weights of 112 mg., an increase of 259 per cent. The grade 0 membrane was absolutely impermeable, as found by an ultrafiltration test with distilled water. No water passed through the membrane during a period of 2 hours at a pressure of 400 mm. of mercury at 21° C.

Increasing amounts of ethylene glycol in the collodion resulted in membranes showing a corresponding increase in weight, as shown in Table I and Figure I.

In Table I the value "G_o"[4] represents the per cent increase of the organo-weight over the dry weight, divided by 100, or grams of organic solvent per gram of nitrocellulose. "G_w"[4] represents the per cent increase of the wet weight over the dry weight, divided by 100, or grams of imbibed water per gram of nitrocellulose. "R" is the ratio of the organic solvent to water.

*"Grade 4" means that the collodion used contained 4 per cent by volume of ethylene glycol in the solvent.

As seen from Figure I the wet weight is directly proportional to the grade of membrane up to and including grade 18. The same is true for the organo-weight from grade 4 to grade 18. The weights of the grade 20 membranes are proportionally less than those of the other grades. For this reason the series was not continued beyond this point.



Reproducibility of Membranes

A series of membranes including grades from 0 to 20 were prepared during the interval from December 21, 1928 to April 16, 1929. No difficulty was experienced in obtaining checks on the wet weights of membranes of the same grade to within ±3

TABLE I.—EFFECT OF ETHYLENE GLYCOL ON WEIGHTS OF MEMBRANES

Average values, in mg.

Membrane Grade*	Dry Weight	Organo-weight	Wet weight	G_o†	G_w‡	R§
0	114	135	124	0.18	0.09	2.10
4	112	630	402	4.63	2.59	1.79
6	113	760	520	5.72	3.60	1.59
8	112	935	625	7.35	4.58	1.60
10	112	1090	750	8.73	5.70	1.53
12	112	1280	886	10.43	6.91	1.51
14	112	1427	1024	11.74	8.14	1.44
16	112	1583	1156	13.13	9.32	1.41
18	112	1751	1237	14.63	10.04	1.46
20	112	1801	1298	15.08	10.59	1.42

*Grade = % by volume of ethylene glycol in the solvent used in making collodion.

$$\dagger G_o = \frac{\text{Organo-weight} - \text{dry weight}}{\text{dry weight}}$$

$$\ddagger G_w = \frac{\text{Wet weight} - \text{dry weight}}{\text{dry weight}}$$

$$\S R = \frac{\text{Organo-weight} - \text{dry weight}}{\text{wet weight} - \text{dry weight}}$$

per cent. When attempts were later made to duplicate these membranes, extremely variable results were obtained. Membranes of grade 6 had organo-weights varying from 809 to 858, and wet weights varying from 583 to 608, as compared with the values given in Table I of 760 and 520 respectively. Similarly, grade 8 membranes had organo-weights varying from 836 to 1101, and wet weights varying from 660 to 880, as compared with the values in Table I of 935 and 625 respectively. The great majority of these variations were in the direction of both higher organo-weights and higher wet weights than those previously obtained, although there was no longer a fixed relationship between the two. These variable results were obtained during the following spring and fall months.

The inconsistencies in most cases could not be attributed directly to the higher temperature, as an increase in temperature would increase the vapor pressure of the alcohol and ether in the collodion films, thereby decreasing the resulting organo- and wet

weights. It seemed probable that the high humidity during the warmer months might lead to irregular results. Both ethylene glycol and alcohol are hygroscopic, and two-thirds of the air which came in contact with the collodion films was enclosed in the chamber under atmospheric conditions. To test the effect of moisture in the films, grade 6 and grade 8 collodion was made up in which the solvent contained 1 per cent by volume of water. This moisture content led to increased weights over the standard values given in Table I. The results are given in Table II.

TABLE II.—VARIABILITY OF MEMBRANES

Weights in mg.

Group	Membrane Grade	Date Made	Organo-weight	Wet Weight
I	6	2/5/29	763	491
	6	2/6/29	757	513
	6	11/4/29	809	608
II	6	11/5/29	858	583
	6	11/6/29	814	583
	6–1*	4/8/30	856	600
III	6–1	4/9/30	862	602
	6–1	4/10/30	810	580
	8	2/12/29	933	615
IV	8	2/13/29	908	600
	8	2/15/29	936	619
	8	9/27/29	1023	860
	8	9/28/29	1008	817
V	8	9/30/29	1101	840
	8	10/1/29	997	775
	8	10/3/29	937	735
	8	10/5/29	1009	713
VI	8–1*	4/18/30	1042	849
	8–1	4/21/30	1053	817

*These membranes were made from collodion containing 1 per cent by volume of water in the solvent.

Groups I and IV in Table II were taken from the original series, which gave good checks. From groups II and III it may be observed that grade 6 membranes made from collodion containing 1 per cent by volume of water in the solvent corresponded quite closely to grade 6 membranes made in November, for which the weights were unduly high. The average increase in the wet weights in groups II and III over those of group I is 17.7 and 17.8 per cent respectively. A great many grade 8 membranes were made. In group V is given a series which shows the ex-

tremely erratic results which may be obtained from day to day. The higher values in group V correspond to those in group VI, which contained added water.

It seems reasonable to assume that the extreme irregularity of results can be attributed to varying amounts of moisture absorbed by the collodion films from the air with which they were in contact.

TABLE III.—REPRODUCIBILITY OF MEMBRANES

Weights in mg.

Membrane Grade	Date Made	Organo-weight	Wet Weights, Membranes No. 1	No. 2	No. 3	No. 4
4	12/27/28	640	410	415		
4	12/28/28	608	405	410	399	
4	11/29/29	664	409	400		
4	12/2/29	616	389	398		
				mean 406± 1.7%		
6	2/5/29	763	484	485	506	488
6	2/6/29	757	513	519	521	511
6	2/7/30	803	535	540		
6	2/8/30	808	544	544		
				mean 522± 3.8%		
8	2/12/29	933	576	606	625	614
8	2/15/29	936	620	624	622	611
8	2/10/30	965	654	656		
8	2/12/30	966	659			
				mean 628± 2.9%		
10	2/21/29	1092	743	752	763	750
10	2/22/29	1102	766	774	784	
10	3/5/30	1115	747	746		
10	3/6/30	1066	756	749		
				mean 755± 1.2%		
12	2/26/29	1242	899	900	904	905
12	4/16/29	1293	917	916		
12	3/15/30	1317	871	858		
12	3/17/30	1246	884	870		
				mean 886± 2.5%		
18	3/20/29	1745	1242	1255	1283	1277
18	3/21/29	1676	1246	1220	1239	1236
18	3/29/30	1791	1237	1229		
18	3/31/30	1793	1229			
				mean 1235± 3.3%		

Membranes made when both humidity and temperature could be kept fairly low, gave the lower weights which correspond to the series made originally. With occasional exceptions membranes made in the winter months of 1930 gave values which checked those of membranes made in the winter months of 1929. The figures given in Table I represent averages of these two groups. The membranes used for ultrafiltration were limited to those having wet weights within ±5 per cent of the values given in Table I.

Not all of the grades of membranes in the series from 0 to 20 were made in 1930, but only those needed for ultrafiltration work. In Table III data are assembled to show the actual reproducibility of membranes made during periods of low atmospheric humidity. Each set of figures shows reproducibility from day to day and from one year to another. Four membranes were made at a time. In some cases all four were weighed. More commonly two were weighed and the rest used for other purposes, or one only might be weighed if it agreed with the expected value. All weighings made on a given set of membranes are included.

In Table III the average deviation from the mean was calculated from membranes number 2. The wet weights of membranes number 1 were not considered to be quite as representative, as these membranes had already been used for obtaining organo-weights. The mean of the average deviations is ±2.6 per cent, which may be used to express the average reproducibility of the membranes under the conditions described.

The Ultrafiltration of Malt Extract

Preparation of Extract

The extract was made by adding 4 parts of cold water to 1 part by weight of ground malt barley. The mixture was shaken by mechanical rotation for 1 hour, decanted into a centrifuge cup, centrifuged at about 2300 r.p.m. with an effective radius of 20 cm., for 10 minutes, and filtered in a refrigerator below 15° C. A slightly turbid brown liquid was obtained. The extract was stored on ice over night and used the next day for ultrafiltration.

Before use the extract was buffered to pH 4.3-4.5, the isoelectric zone of malt amylase.[22] An acetic acid-sodium acetate mixture was selected for buffering, due to its effectiveness over a range of pH 3.5 to 6.[23] It has been recommended particularly for use with malt amylase.[24] The concentration of buffer needed was determined by titrating the extract electrometrically with 0.1 molar acetic acid. The proteins in the extract themselves have a buffering action. At a pH of 4.4 the mixture was about 0.02

molar with respect to acetic acid. As it seemed desirable to keep the concentration of added salt to a minimum, the proportion of the two buffer constituents to be used was determined by titrating an extract made 0.03 molar with acetic acid against that made 0.03 molar with sodium acetate.

Several samples of fresh unbuffered extract gave pH values of 5.7 to 5.9. This changed quite rapidly with time. An extract having a pH value of 5.9 on the day prepared, after standing three days in the refrigerator gave a pH value of 3.9 and was turbid in appearance. Buffering to pH 4.4 also causes turbidity.

Just before ultrafiltering, a total of 1.5 cc. of 1.0 molar acetic acid and 1.0 molar sodium acetate solutions, in the proportion determined, was put into a 50 cc. volumetric flask, diluted to 50 cc. with the cold extract and mixed.

Ultrafiltration was selected rather than dialysis, as it was planned to test quantitatively the liquid on each side of the membrane for diastatic activity. A dilute solution of enzyme material is less stable than a concentrated solution, which is attributed to hydrolysis.[28] This suggests that in ordinary dialysis, negative tests for active enzyme in the diffusate would be inconclusive, as the volume of diffusate is usually large with respect to the volume of the dialyzed solution. In ultrafiltration the total volume of solution is unchanged.

The ultrafilter was similar to that designed by Pierce.[7] The membrane was supported by a gold-plated wire gauze, which was separated from a cut-off Erlenmeyer flask above, and an ordinary porcelain funnel below, by rubber gaskets. The parts were clamped together securely. Forty cc. of buffered extract were put in the ultrafilter, the latter adjusted in a shaking machine, and a pressure of 300 mm. of mercury applied. The increased pressure was created by connecting the ultrafilter to a nitrogen tank and manometer. Nitrogen was used because of its inert character.

Thirty cc. of filtrate, three-fourths of the volume of extract used, were caught in 6 successive 5 cc. portions. Enzyme activity was determined by the gravimetric method of Sherman, Kendall and Clark.[25] Unless otherwise stated, activities are calculated according to their scale. The starch solution was buffered to a pH of 4.5[26] with 0.01 molar acetate.[24] Ordinarily the activity was determined on 0.04 cc. of the original extract, 0.5 cc. of each of the 6 filtrates, and 0.02 cc. of the residue. Portions of the original and residue were diluted and aliquots taken for the determination. A smaller portion was necessary if any of these was unusually active. Total solids, used in calculating the diastatic power, were determined by evaporating to dryness 2 cc. of each of the 8 fractions, at 70° C., the temperature varying roughly $\pm 2°$ C.

By using an extract prepared fresh each time under standard conditions, the diastatic activity did not differ greatly from one time to another. The mean diastatic power for 12 extracts prepared as described, was 38.1, with an average deviation of ±1.07, or ±2.8 per cent. In view of the nature of the process, such an experimental error may be considered reasonable.

Determinations of hydrogen ion activity on the original, the filtrates, and the residue, showed no large differences, all of them being within the limits pH 4.3-4.5.

Results with Malt Extract

The filtrates were always clear and only slightly colored, the residue was always turbid and contained a precipitate which settled out. Qualitative color tests were made on several occasions. In each case all six portions of the filtrate gave positive Molisch and positive xanthoproteic tests. In each case the original extract and the residue gave positive biuret tests. Filtrates which did not contain active enzyme gave negative biuret tests. Filtrates containing active enzyme in some cases gave a doubtful biuret test. A filtrate, 0.2 cc. of which precipitated 113 mg. of cuprous oxide upon Fehling digestion, gave a blue color with the biuret reagent. A filtrate, 0.1 cc. of which precipitated 355 mg. of cuprous oxide gave a violet color, indicating the presence of protein. The filtrates were not concentrated for these tests.

The time required to collect each successive 5 cc. portion of filtrate increased progressively. For example, with a grade of 10 membrane, the first portion was collected in 12.5 minutes, the sixth portion in 31 minutes. The time is shorter with more permeable membranes and longer with less permeable. The solid content of 12 samples of extract prepared under like conditions was 42.8 ±1.0 mg. per cc. The average deviation is ±2.3 per cent. The filtrates showed a gradual increase in total solids with the volume of liquid filtered, as shown in the following table:

TABLE IV.—TOTAL SOLIDS

In mg. per cc.

Membrane Grade	Original Extract	Filtrates I	II	III	IV	V	VI	Residue
8	42.2	34.2	36.2	36.2	36.9	37.3	37.5	54.2
10	42.2	35.1	36.7	36.7	37.4	37.6	38.6	47.5
12	41.7	34.0	36.9	36.9	37.6	38.0	39.1	44.0

As may be seen from Table IV, when the original extracts are very similar in their solid content, differences in the permeability

of membranes of different grades can be demonstrated by the amount of material passing through them. Increasing amounts of dissolved substances were carried through the membranes as the grade increased, except in the case of filtrate I. Confirmatory evidence of the increase in permeability is furnished by the amount of material left in the residue, which decreased as the grade of membrane increased. The differences involved are so small that only those cases can be compared where the solid content of the original extracts varied by about 1 per cent or less. The gradual increase in the amount of material passing through the membrane with the time of filtration is probably due to the increase in the concentration of the material being filtered.

Membranes up to and including grade 8 were impermeable to the enzyme under the conditions described. Membranes of grade 9 and over were permeable. With a membrane permeable to the enzyme a sudden increase in activity was observed in the fifth or sixth filtrate. In all cases the first four filtrates yielded in the neighborhood of 50 mg. of cuprous oxide per 0.5 cc. The most moderate increase in activity was from 53 mg. of cuprous oxide from 0.5 cc. of the fourth filtrate to 111 mg. from 0.5 cc. of the fifth. The former value was the actual blank for the filtrate. This was shown by inactivating the enzyme by boiling samples before the activity determinations were made. The presence of reducing sugars in the filtrates is evident, as a blank on the reagents used ordinarily yielded 37 mg. of cuprous oxide.

As temperature could not be accurately controlled, four filtration tests were made to study the effect of a change in temperature. The results are given in Table V. Grade 9 membranes all made at one time were used. A moderate increase in temperature did not cause the enzyme to pass through the membrane sooner, but once started, it passed through more rapidly.

No enzyme activity was shown in any case by the first five filtrates, that is, by 25 cc. of filtrate. In all cases the sixth filtrate showed activity. These last results are very irregular from a

TABLE V.—EFFECT OF TEMPERATURE ON PERMEABILITY

Activity expressed as mg. Cu_2O

Temperature	Original Extract	Filtrates I-V inclusive	Filtrate VI	Residue
	0.04 cc.	0.5 cc.	0.5 cc.	0.02 cc.
19–20.5° C.	203	0	267	218
19.5–20	202	0	170	218
24–24.5	206	0	495+ over-reduced	183
24–26	213	0	353	182

quantitative point of view. This is because the last of the filtrate caught contained active enzyme material and a small difference in volume, even a drop, at this point, would case a considerable difference in the activity determination of the filtrate. A drop would not make an appreciable difference in the 10 cc. of residue. The two residues at 20°, and at 25°, gave exceptionally good checks. The residues obtained at 20° were more active than those obtained at 25°. The filtrates at 20° were somewhat less active than those at 25°. These results indicate that an increase of 5° throughout the period of ultrafiltration causes an increase in the amount of enzyme material passing through a membrane. The rate of filtration was also changed. At 25° the total time of filtration averaged 123 minutes, at 20°, 143 minutes. The temperature should therefore be kept as constant as possible. The procedure adopted was to keep the liquid chilled until it was put in the ultrafilter, after which the temperature could be controlled only roughly. The temperature of ultrafiltration was in the neighborhood of 20° C. This varied as much as ±3°, but not throughout the period of ultrafiltration.

A rough calculation of the recovery of enzyme activity may be made by calculating the original activity of the 40 cc. of extract put in the ultrafilter and comparing with the total activity of the filtrates plus that of the residue, assuming the latter to be 10 cc. These calculations are given in Table VI.

TABLE VI.—EFFECT OF PERMEABILITY ON ENZYME RECOVERY

Activity expressed as mg. Cu_2O

Membrane Grade	Original Extract	Filtrates	Residue	Total Recovered	Per cent Recovery
	40 cc.	30 cc.	10 cc.		
8	205000	0	201500	201500	100
8	202000	0	141000	141000	70
9	202000	1860	109000	110860	55
10	199000	3200	90000	93200	47
14	197000	7820	75000	82820	42
12	199000	13530	64000	77530	39

The high permeability of the grade 12 membrane could not be accounted for, as its wet weight was between those of membranes of grade 10 and 14. In Table VI it is shown that as more of the enzyme passes through a membrane, more of it is lost, due to adsorption in the membrane, dilution as the enzyme first appears in the filtrate, or both. Although the first 30 cc. of filtrate through grade 8 membranes showed no diastatic activity, variable results were obtained in calculating the recovery of total activity. This

is probably because these membranes were near the borderline of permeability under the conditions described.

Since in no case was enzyme activity obtained in the first 20 cc. of filtrate, it was thought that it might be necessary for the pores of the membrane to become saturated with enzyme material before the latter could pass through. A grade 8 membrane similar to one found impermeable to the enzyme under standard conditions, was selected for use in studying this point. Forty cc. of buffered extract were put in the ultrafilter and 2 portions of 15 cc. each of filtrate were caught. The residue was then poured out as completely as possible and a second 40 cc. portion of buffered extract immediately introduced. Thirty cc. of filtrate were caught in two 10 cc. portions and two 5 cc. portions. The original, the 6 filtrates, and the 2 residues were tested for activity. The first 30 cc. of filtrate showed no enzyme activity, after which a gradual increase in activity was observed in the succeeding portions of filtrate. The inactive character of the first 30 cc. of filtrate confirmed earlier results. The two residues had the same activity, 0.02 cc. yielding 282 and 279 mg. of cuprous oxide, respectively. Their activity, based on solid content, was nearly three times that of the original.

Similar experiments were performed with grade 6 membranes, also impermeable to the amylase as previously tested. In the first test (Case I) 80 cc. of buffered extract were put immediately into the ultrafilter, in the second test (Case II), two 40 cc. portions were used successively. The same number and volume of filtrates were collected as in the previous experiment. In both cases the first 30 cc. of filtrate showed no activity. The following portions showed a gradual increase in activity. The activity of the residues was approximately the same in each case. The weight of cuprous oxide obtained from 0.02 cc. of the first residue (Case I), amounted to 309 mg. The weights obtained from 0.02 cc. of each of the other two residues (Case II), were 304 and 323 mg. respectively. Since the two residues obtained by successive filtrations through the same membrane had nearly the same activity, although the first 30 cc. of filtrate showed no enzymic activity and the second 30 cc. did, it appears that a certain amount of enzyme is lost by adsorption in the membrane.

By the use of relatively impermeable membranes enzyme activity was increased by ultrafiltration, in terms of solid content, to about three times that of the original extract. Ultrafiltration with grade 8 membranes, under conditions such that the filtrates were inactive, caused an increase in enzyme activity from 37.5 to 122 and from 38.4 to 124. The recovery of enzyme activity, as calculated in Table VI, was 100 per cent in each of these cases.

Since it had been found that a grade 6 membrane was impermeable to the enzyme when 30 cc. of extract (1:4) were ultrafiltered through it, but permeable when 60 cc. were ultrafiltered, it seemed advisable to try the effect of increasing the concentration of the extract. Accordingly 40 cc. of an extract prepared from 1 part by weight of ground barley malt to 2.5 parts of water were used in the ultrafilter with grade 6 membranes. Active enzyme was present in the fourth, fifth, and sixth portions of filtrate. Grade 4 membranes were also tested and found impermeable under the conditions described, both when 30 cc. and 60 cc. of filtrate were caught. The time required for the ultrafiltration of 60 cc. of extract (1:2.5) was 12 hours, the last 5 cc. portion required 1.5 hours. From this one may conclude that grade 4 membranes are impermeable to the enzyme, but grade 6 membranes, as well as those of higher grades, are permeable if enough enzyme passes through to saturate the membrane pores. The activity was increased by ultrafiltration of the concentrated extract (1:2.5) through grade 4 membranes from 38.8 to 110, 42 to 140, and 39.5 to 126. The recovery of enzyme activity was 100 per cent in each case.

Although considerable purification can be effected by the ultrafiltration of the extract, the more active residue becomes very turbid, deposits a precipitate on the membrane, and is somewhat colored. It therefore seemed desirable to study the ultrafiltration of enzyme material in a somewhat purer state than in the extract.

The Ultrafiltration of Purified Enzyme

Purification

An extract was prepared, using 1 part by weight of ground malt to 2.5 parts of water. This was shaken an hour, centrifuged 15 minutes at about 2300 r.p.m. with an effective radius of about 20 cm., and filtered in the ice box. Ammonium sulfate was added to the filtrate in the proportion of 45 grams to 100 cc.[15] When the sulfate had dissolved, the mixture was centrifuged for 10 minutes and the supernatant liquid discarded. The protein material obtained was stirred up with 50 cc. of water and let stand over night. In the morning a precipitate was observed. Water was added until the volume equalled that of the original filtered extract. The mixture was still very turbid. A portion of this was buffered and 40 cc. were put in the ultrafilter as in the previous work.

A grade 4 membrane was used, as it was desired to permit the passage of ammonium sulfate but not of enzyme material. The activity of the original suspension was 40.5, which is only slightly greater than the average value of 39.7 for the untreated extract

(1:2.5). Apparently enough ammonium sulfate was carried down by the precipitate to compensate for the carbohydrates removed during the precipitation treatment, or the sulfate present may have decreased the activity of the amylase. The original suspension, the six 5 cc. portions of filtrate, and the residue, gave positive tests for sulfate ion. The filtrates showed no enzymic activity. The activity of the residue was 140. Neither a clear solution nor a decided increase in activity, compared with that of the extract, resulted from the ammonium sulfate treatment, before or after ultrafiltration. Ultrafiltration is not a satisfactory method for freeing a liquid from a relatively high concentration of electrolyte.

Certain investigators consider malt amylase to be closely related to a complex protein, of the nature of an albumin combined with a proteose or peptone.[14, 28] A qualitative test was next made, applying the principle of Sorensen[27] in obtaining crystalline egg albumin, that salts and carbohydrates remain dissolved, while globulins are precipitated by a half-saturated solution of ammonium sulfate, and albumin by a saturated solution. It was thought that the enzyme might be isolated with the albumin fraction thus separated. To a portion of filtered malt extract (1:2.5) was added an equal volume of a saturated solution of ammonium sulfate. The precipitated globulins were separated by centrifuging. The supernatant liquid was poured off and both parts saved. Crystals of ammonium sulfate were added to the liquid until they no longer dissolved. The precipitated albumin was separated by centrifuging and saved. The two protein precipitates were tested roughly for activity, as well as the last supernatant liquid. The only fraction which showed considerable enzyme activity was the first precipitate. This suggests the possibility that the enzyme may be carried down with any protein precipitate. This point might be worth investigating by those interested in studying the nature of the enzyme.

Alcohol precipitation, omitting dialysis, was next tried. Filtered malt extract (1:2.5) was diluted 1:1 with cold alcohol and let stand 15 minutes with occasional stirring. The mixture was centrifuged for 5 minutes at about 2300 r.p.m. with an effective radius of 20 cm., and the liquid decanted off. This was brought to an alcohol concentration of 65 per cent by volume, which gave the fraction used by Sherman and Schlesinger.[28] It was allowed to stand 15 minutes with occasional stirring. It was centrifuged for 5 minutes and the supernatant liquid discarded. The gummy precipitate was dried in a desiccator. When dry it was ground to a fine powder. This process was carried out twice. The activities of the products were 360 and 374, compared with 39.7, the aver-

age value for the concentrated extract (1:2.5), or 38.1 for a more dilute extract (1:4). This process can be carried out completely in 3.5 hours.

Results with Purified Enzyme

For ultrafiltration tests 20 mg., ordinarily, of dry enzyme material were rubbed up with a small amount of water, buffered to pH 4.4 with 0.01 molar acetate buffer, and diluted to 50 cc. A somewhat milky suspension resulted, in which undissolved particles were visible.

Membranes of grade 4, 6, 8, 9, 10, 12 and 18, were tested for permeability under the same conditions of ultrafiltration as used previously. Ordinarily 0.5 cc. of the original suspension, 4 cc. of each portion of filtrate, and 0.15 cc. of residue were used in making the activity determinations. The membranes were all found to be impermeable. The residues showed approximately the same activity as the originals, showing that no further purification resulted from ultrafiltration. The rate of filtration was much greater than with the extract. The time varied from about 90 minutes with the less permeable membranes to 30 minutes with the more permeable ones.

To see if increased concentration might produce a different result, 100 mg. of enzyme material in 50 cc. were used with a grade 9 membrane, such as was found permeable in the case of the dilute extract. No activity was exhibited by the filtrates. The volume of liquid filtered was increased. In the case of the extract, the second 30 cc. portion of filtrate contained increasing amounts of active enzyme, when grade 6 and grade 8 membranes were used. A similar experiment was conducted with a grade 8 membrane, using 40 mg. of enzyme material in 100 cc. Of this, 80 cc. were put in the ultrafilter and 60 cc. of filtrate caught and tested. No enzyme activity was shown by any portion of the filtrate.

The enzyme solution was buffered to a pH value of 5.5, the concentration being 20 mg. in 50 cc., and ultrafiltered through grade 12 and grade 18 membranes. It was thought that the enzyme would be more highly dispersed on the alkaline side of its iso-electric point and that globulins present would also be more highly dispersed. The filtrates showed no activity.

When working with malt extract it was observed that in nearly all the cases where the filtrates were found to be inactive, the residues showed 100 per cent recovery of activity. When the filtrates contained active enzyme the recovery of active material was always considerably less than 100 per cent. Adsorption on the surface of the membrane alone seems to be negligible, while in the membrane pores, where the surface of contact is very great, ad-

sorption increases correspondingly. The same method of calculation as was used in Table VI was applied to the results with the purified enzyme. This is given in Table VII. The filtrates showed no activity.

Although the calculations given in Table VII are very rough, they show that a fairly large proportion of enzyme activity is lost by ultrafiltration through the more permeable membranes. The two experiments carried out at pH 5.5 show an exceptionally large loss of activity. This was not due to inactivation of the enzyme, as the original solutions were tested at the same time as the residues and showed no such effect. All of the enzyme lost may have been adsorbed by the membrane, or if any passed into the filtrate it may have been completely hydrolyzed due to its very low concentration.

TABLE VII.—ENZYME RECOVERY IN THE ULTRAFILTRATION OF THE PURIFIED PRODUCT

Activity expressed as mg. Cu_2O

Membrane Grade	Concentration of Enzyme	Activity of Original Suspension	Activity of Residue	Per cent Recovery
	mg. per 50 cc.	40 cc.	10 cc.	
4	10	8880	10280	100
4	10	8240	8480	100
4	20	17120	18666	100
4	30	27771	26500	100
6	10	8240	7920	100
6	10	7840	7280	100
8	20	16240	19000	100
8	20	18400	17100	100
9	20	16160	10270	63
9	20	15840	11800	75
9	100	82200	61600	76
10	20	14560	11266	77
12	20	15040	9200	61
12	20	14400	8450	59
12	20	14880	3900	26*
18	20	14400	7600	53
18	20	15600	4000	26*

*In these two cases the original suspensions were buffered to pH 5.5 instead of 4.4.

Adsorption Tests

To test further the validity of the conclusion reached that a membrane is able to adsorb active enzyme material, tests were made in which 20 mg. of the purified product were rubbed up with water, buffered to give a pH value of 4.4 and diluted to 50 cc. In the first instance half of this was put in one tube as a blank experiment and half put in a second tube in contact with a grade 10

membrane. The tubes were kept cold and shaken occasionally. After 24 hours 1 cc. of the blank solution precipitated 360 mg. of cuprous oxide. The portion which had been in contact with a membrane for the same length of time had lost 73 per cent of its activity. This indicates that malt amylase, like protein material,[29] is adsorbed by nitrocellulose membranes.

Similar tests were made in which one portion of enzyme solution was kept as a blank, a second portion was placed in contact with a membrane, and a third portion was placed in contact with a membrane which had been previously soaked in a 0.1 per cent solution of egg albumin. The three solutions were tested for diastatic activity after standing for 1 day and for 4 days in a refrigerator, with occasional shaking. Two such experiments were made. Grade 12 membranes were used. The results are given in Table VIII.

TABLE VIII.—ADSORPTION OF ENZYME BY MEMBRANES

Activity expressed as mg. Cu_2O per cc. of solution

	Test	Activity after 1 day	% loss	Activity after 4 days	% loss
Control enzyme solution	I	355	..	335	6
	II	394	..	384	3
Enzyme solution in contact with untreated membrane	I	120	66	26	92
	II	186	53	37	90
Enzyme solution in contact with membrane treated with albumin solution	I	214	40	104	69
	II	263	33	130	66

In the third column of the table is given the per cent loss of activity of the enzyme solution in contact with both untreated and treated membranes, calculated from the activity of the 1-day old control enzyme solutions. In column 5 the first two numbers give the loss of activity of the control enzyme solutions after standing 3 days longer than in the first determinations. The succeeding numbers in column 5 give the per cent loss of activity based on the 4-day control.

The tubes were shaken vigorously immediately before removing samples for testing. As may be seen from the table, a large proportion of the enzyme activity is lost by contact with a membrane. This loss is significantly less if the membrane has first been soaked in a solution of egg albumin. From this it is concluded that the enzyme is adsorbed by a nitrocellulose membrane in the same way that egg albumin is adsorbed.

It seemed possible that a membrane pretreated with a protein solution might permit the diffusion of the enzyme. A grade 12 membrane was soaked in a solution of egg albumin over night and then tested for permeability. No active material was detected in the filtrates.

Conclusions

1. Nitrocellulose membranes graded by means of ethylene glycol, have organo-weights and wet weights which are proportional to the amount of ethylene glycol in the collodion, over a range from grades 4 to 18 inclusive. Such membranes may be prepared by evaporation for a period of 6 hours, followed by washing in water over night. The membranes are ready for use in one-third the time required by the procedure of Pierce.[7] High atmospheric humidity causes the membrane weights to be increased. During periods of low temperature and low humidity, membranes can be reproduced with an average variation of ± 2.6 per cent in wet weight. For prolonged work with these membranes, equipment should be provided for controlling temperature and humidity.

2. That the permeability of a membrane corresponds to its grade, may be demonstrated by a determination of total solids on the filtrates obtained from the ultrafiltration of malt extract. A membrane of a certain grade permits the passage through it of a greater amount of dissolved substances than a membrane of a lower grade.

3. The rate of ultrafiltration is increased by an increase in temperature. A difference of 5° C. throughout the period of ultrafiltration causes a membrane to be somewhat more permeable to malt amylase at the higher temperature.

4. As more active enzyme passes into the filtrates, less of the total activity is recovered. This may be attributed to adsorption of the enzyme in the membrane, and possibly also to hydrolytic decomposition due to dilution in the filtrate.

5. The permeability of a membrane to malt amylase is dependent to a certain extent on the conditions of ultrafiltration. At a temperature of about 20° C. and a pressure of 300 mm. of mercury, no active enzyme is detected in the first 30 cc. of filtrate, using 40 cc. of malt extract (1:4) with grade 6 and grade 8 membranes. Other conditions being the same, the filtrate obtained with a grade 9 membrane contains active enzyme. If the volume of malt extract (1:4) is increased to 80 cc., the first 30 cc. of filtrate is inactive, but the succeeding 30 cc. of filtrate contain active enzyme, with both grade 6 and grade 8 membranes. If the concentration of the extract is increased to 1:2.5, the first 30 cc.

of filtrate contain active enzyme, using grade 6 membranes. Grade 4 membranes are impermeable to the enzyme under all of the conditions studied. In every case in which active enzyme is found in the filtrate, considerably less than 100 per cent of the original activity is recoverable. In most cases in which the filtrate is inactive, 100 per cent of the activity can be accounted for in the residue. These results indicate that a definite amount of enzyme is removed from the liquid in its passage through a membrane. The thickness and porosity of a membrane increase with an increase in grade. The amount of enzyme removed in passing through a membrane may be expected to increase as the membrane thickness becomes greater. As the membrane grade decreases and the nitrocellulose gel becomes more compact, a point may be reached where the size of the pores becomes too small to permit the entrance of enzyme material.[30]

6. Malt extract may be purified considerably by ultrafiltration by the use of membranes impermeable to the enzyme but permeable to more highly dispersed substances. Qualitative color tests indicate that these are carbohydrates and amino acids. The portion remaining under these conditions is about 3 times as active, in terms of its solid content, as the original extract.

7. The material present in malt extract, remaining in solution in 50 per cent, but precipitated by 65 per cent alcohol, was 9 times as active, in terms of solid content, as the original extract. This purification process requires only 3.5 hours, including the preparation of the extract. Such a product is not suitable for ultrafiltration experiments, since 30 cc. of filtrate shows no activity with a grade 18 membrane, using 20 mg. of dry product in 50 cc.

8. Enzyme material is adsorbed by nitrocellulose membranes in the same way that egg albumin is adsorbed. This confirms the conclusion that adsorption of enzyme occurred during ultrafiltration when the conditions were such that the membranes were permeable to the enzyme. Adsorption would be expected to be greater during the passage of enzyme material through a membrane because of the much greater membrane surface with which it comes in contact.

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